ANTIGENIC RELATIONSHIP OF DIFFERENT PORCINE ROTAVIRUS ISOLATES AND THE IDENTIFICATION OF A NEW PORCINE STRAIN

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An abstract of a Thesis by
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The problem. Currently, only one strain of porcine rotavirus has been reported. This strain, designated OSU, has been attenuated and is currently available as a federally licensed, live oral vaccine for pigs. This study is concerned with the antigenic relationship of the OSU vaccine strain to four virulent porcine rotavirus isolates as measured by the vaccine's serologic cross-reactivity in vitro.

Procedures. Four virulent isolates of porcine rotavirus were adapted to tissue culture. Purified viral antigens were prepared from low passage tissue culture adapted virus fluids. Upon purification both complete and single-shelled rotavirus particles were observed by electron microscopy. Furthermore, all virus bulks were identified as rotavirus by positive fluorescence obtained by immunofluorescent staining of inoculated cell cultures. The antigens were used to produce high titer hyperimmune sera in guinea pigs. These sera were used in appropriate serological studies in vitro to determine the amount of antigenic cross-reactivity between the different isolates. Antigenic cross-reactivity was measured by the amount of virus neutralized by homologous and heterologous sera. Neutralizing indices were calculated and all virus isolates compared.

Findings. Two distinct serotypic groups were observed; the original OSU strain and a new strain of porcine rotavirus identified as the Iowa strain. The new strain was found to be significantly different from the OSU strain as measured by its lack of serologic cross-neutralization.

Conclusions. The results indicate a minor antigenic similarity between the two strains as found in low dilutions of the hyperimmune sera. Nevertheless, there was an absence of any major antigenic similarity between the two strains. Therefore, this lack of any major antigenic cross-neutralization between the two strains suggests the Iowa strain is unique and can be classified as a new strain of porcine rotavirus.

TABLE OF CONTENTS

LIST OF TABLES

INTRODUCTION

Within the last eight years rotavirus has been found to be an important etiological agent of acute gastroenteritis of nursing and weanling pigs (Bohl et al. 1978; Bridger 1980). In 1975 a reovirus-like particle was isolated from a pig with a clinical history of watery diarrhea (Lecce et al. 1976; Woode et al. 1976a). Since this time rotavirus isolation by different laboratories has increased as better virus isolation procedures have been developed.

Rotavirus is ubiquitous and has been isolated from a wide variety of domestic animals with scour problems (McNulty, 1978). When an acute infection occurs, virus is shed in the feces in large numbers with virus titers approaching 10 Log_{10} $TCID_{50}$ per gram of feces (Woode 1978). The virus in fecal matter is quite stable to both temperature and various disinfectants and therefore it is not easily eliminated from the environment (Estes et al. 1979; Snodgrass and Herring 1977). Both the high number of virions shed in the feces and the exceptional stability of the virus accounts for its widespread prevalence in swine herds.

Rotavirus isolates are morphologically indistinguishable from one another by electron microscopic examination (Woode et al. 1976b). The rotavirus capsomere is icosahedrally arranged and resembles a wheel with spokes

protruding into the center (Flewett et al. 1974). The Latin meaning of Rota is wheel, and the generic name rotavirus was adopted by the Reoviridae working team of the World Health Organization in 1978 (Derbyshire and Woode 1978). Rotavirus belongs to the family Reoviridae which also includes orbiviruses and reoviruses. When examined by electron microscopy, rotavirus and reovirus are morphologically similar. Nevertheless, serologic studies indicate the two will not cross-react and therefore possess different antigenic characteristics (Welch and Twiehaus 1973).

Two types of virus particles are associated with rotavirus infections. Complete particles are 70 nm in size and have a double layered capsid with a hexagonal core of 38 nm. The complete particle has a buoyant density of 1.36 g/ml and is considered to be the infectious particle (Flewett and Woode 1978). A small single-shelled virus particle, 60 nm in size, is also found. Its origin may be due to the uncoating of complete particles. These single-shelled particles have been found to be non-infectious and have a buoyant density of 1.38 g/ml (Rodger et al. 1977). The rotavirus genome consists of eleven segments of dougle stranded RNA of various molecular weights (Rodger et al. 1975). The double-shelled particle consists of seven to ten polypeptides whereas the single-shelled particle only contains four or five (Rodger et al. 1977).

Studies have shown that antigens for complement fixation, fluorescent antibody and immune electron microscopy are group specific and are associated with the inner capsid layer (Flewett 1976; Woode et al. 1976b). The neutralizing and haemagglutinating antigens are species specific and are associated with the outer capsid layer (Bridger 1978).

Fluorescent antibody studies conducted on tissue sections from infected pigs have demonstrated that once rotavirus has entered the gastrointestinal tract it has a predilection for the distal portion of the mature epithelial cells of the villi which line the small intestine (Crouch and Woode 1978; Mebus 1976). Cells which line the crypts of the villi have not been found to contain rotavirus (Theil et al. 1978). Early viral replication results in cytoplasmic vacuolization and cell death. Dead cells will slough off into the lumen of the gut and a reduction in the length of the villi is seen throughout the small intestine (Mebus et al. 1971). The villi become club-shaped or blunted and this condition is known as villus atrophy (Dubourguier et al. 1978; Theil et al. 1978). As the epithelial cells are lost both the absorptive and digestive capabilities of the small intestine are impaired (Flewett and Woode 1978) and a diarrhea ensues. Eventually, the damaged epithelial cells are replaced with immature, less susceptible cells from the crypts resulting in a self-limiting disease. A hyperplasia of the small intestinal

crypt epithelium has been seen in studies done with diarrheic pigs (Middleton 1978; Theil et al. 1978). The incubation period prior to the onset of disease is short, ranging from 18 to 72 hours. The predominant clinical signs consist of vomition, anorexia, depression and watery diarrhea, which many times contains yellow or white flecks of undigested curd (Theil et al. 1978). If the pig becomes dehydrated during infection, acidosis and death will ensue. However, not all procine rotavirus infections induce a clinical response, as subclinical infections are well documented and have been detected by viral shedding in the feces of diarrheal-free baby pigs (Woode and Crouch 1978). Mortality in rotavirus infections is low when compared to other porcine enteric diseases such as transmissible gastroenteritis (TGE). Nevertheless, the stability of the virus and the high titer observed in the feces of infected pigs, contribute to a persisting high morbidity especially in weanling pigs. All sows and gilts tested have rotavirus antibody in the sow's milk which provides passive immunity to the nursing pigs. When milk antibody levels decrease substantially, in about two to three weeks post-farrowing, the passive protection of nursing pigs is reduced (Bridger 1980). It is postulated, that the stress associated with weaning and the loss of passive immunity will result in a compromised pig ready for rotavirus infection (Lecce and King 1978).

Concurrent infections are known to occur where rotavirus has previously been diagnosed. Some of the most common agents that have been associated with rotavirus in these coinfections are: TGE, Escherichia coli and Isospora suis. Increased morbidity and mortality are seen in pigs when any of these enteric pathogens are present in conjunction with virulent rotavirus (Dubourguier et al. 1978; Roberts and Walker 1982; Theil et al. 1979; McNulty 1978).

The inconsistency found in trying to reproduce rotavirus infections in conventionally reared pigs has lead researchers to the use of gnotobiotic pigs. Pigs are taken from the uterus by cesarean section and are aseptically transferred to germ-free bubbles. Here rotavirus infections can easily be reproduced. Work done in this area has revealed that pigs under seven days of age are the most susceptible and that older animals will tend to recover from the infection faster. Older gnotobiotic animals also may only develop a subclinical infection, thus making the younger animal the test system of choice (Woode and Crouch 1978).

The delay in recognition of rotavirus as a major enteric pathogen has been related to the difficulty in isolating and replicating the virus in tissue culture (Thouless et al. 1977; Woode et al. 1976a). Cell cultures could be infected with virus, however, infectivity was usually lost after 3 or 4 passes (Babiuk et al. 1977; Wyatt

et al. 1974). A major breakthrough in the in vitro cultivation of rotavirus occurred in 1977, when it was discovered that the porcine strain of rotavirus could be successfully passed in tissue culture if the viral suspensions were treated with pancreatic enzymes (Theil et al. 1977). Since this discovery, trypsin has been reported as the most important enzyme in pancreatin for increased rotavirus replication. Trypsin also has a direct action on the virus and not the cells in which the virus is grown (Barnett et al. 1979; Graham and Estes 1980). Nevertheless, trypsin is required in the medium for successful passage of most rotaviruses and seems to be required for maturation of complete, infectious particles.

Since all domestic animals demonstrated some level of antibody to rotavirus, sera used in growth media for cell cultures were examined for their effect upon rotavirus replication. It was found that non-specific serum inhibitors were in low concentrations of all sera tested (Sato et al. 1980). Even gamma globulin free sera contain these non-specific inhibitors (Tokuhisa 1981). Thus the cells must be laboriously rinsed prior to inoculation. The rinsing of the cells and the incorporation of subtoxic concentrations of trypsin in the cell media have presented researchers with a tool for a rapid diagnosis of rotavirus infections by immunofluorescent staining of inoculated cell cultures.

Studies have been done to compare the sensitivities

of various diagnostic tests such as: electron microscopy (EM), fluorescent antibody (FA), enzyme-linked immunosorbent assay (ELISA), haemagglutination (HA) and immunodiffusion. The results indicate that immunofluorescent staining of inoculated cell cultures is the most sensitive procedure (Akskaa and Bloch 1981; Ellens et al. 1978; Rhodes et al. 1978). Nevertheless, this test system will only detect group antigens and thus can only be used as a diagnostic technique.

With the ability to grow rotaviruses in tissue culture, serological studies have been initiated to demonstrate the relationship of different rotavirus isolates. Neutralizing antibody is the important parameter to evaluate when comparing isolates as it is considered to be species specific, and will be directly related to any cross-protection seen in vivo (Woode et al. 1982). Three human strains of rotavirus have been identified (Wyatt et al. 1982) and further work suggests rotavirus isolates may demonstrate different degrees of serotypic relationship (Woode et al. 1982). The OSU strain of porcine rotavirus is currently the only strain reported in pigs.

Several virulent field strains of porcine rotavirus were isolated from clinically sick pigs. In this study, the relationship of these isolates to the OSU strain was determined using appropriate serological techniques. The use of high titer hyperimmune sera in this study was

essential because the non-specific inhibitors found in a normal serum would present false data. Thus, by using a high dilution of serum the non-specific inhibitors, which are low in titer, could be eliminated. This allowed for the proper evaluation of the antigenic relatedness of the isolates to the attenuated OSU-vaccine strain.

MATERIAL AND METHODS

Cell Cultures. Swine testicular (ST) and embryonic rhesus monkey kidney (Ma-104) cell lines were used to pass and assay the rotavirus isolates. Cell passage was restricted to the 70 to 90 passage levels for both cell lines. Mycoplasma, bacterial and fungal tests were run on each tenth passage of the cells and all cell passes were free of extraneous agents throughout the study. Cells were grown in four types of containers: (1) 690 cm^2 roller bottles (Corning), (2) 25 cm^2 plastic flasks (Corning), (3) 96-well, flat-bottom microtiter plates (Linbro) and (4) Leighton tubes with floating coverslips (Corning). For starting cell cultures, 30 x 10^6 cells per roller bottle, 2 x 10^6 cells per 25 cm^2 flask, 5 x 10^4 cells per well in the plates and 3 x 10^5 cells per coverslip were found to be optimal for the development of a confluent monolayer within four to five days after seeding. The growth medium consisted of Eagle's MEM with nonessential amino acids, L-glutamine and 10% tissue culture grade calf serum (KC Biological). The medium was pH adjusted to 7.4 with 5%

sodium bicarbonate and supplemented with a 50 mcg/ml concentration of gentamicin (Schering). Confluent cell monolayers were maintained with the above medium with the exception of calf serum concentration which was dropped to 2%. To pass the cells in culture, the maintenance medium was removed from confluent roller bottles. The monolayer was washed once with 20 ml of a trypsin (ICN Laboratories) versene (Fisher) medium. The trypsin-versene (TVM) medium consisted of a 500 mcg/ml concentration of trypsin in versene buffer solution. The TVM was then pH adjusted to 7.8 with a 5% sodium bicarbonate solution. After rinsing, a fresh 20 ml of TVM was added to each bottle and the bottles were held at 37°C until the cells started to detach from the glass. The cell suspension was collected and the roller bottle was rinsed twice with 20 ml of growth medium. The growth medium rinses were added to the subcultured cells which were centrifuged at 200 x g for 10 minutes. The supernatant was removed and the cell pellet was resuspended in fresh growth medium. A viable cell count was done in a hemacytometer. Cells were resuspended in fresh growth medium and distributed into appropriate cell containers. The cells were allowed to reach confluency at 37°C in a tight-cap closed system.

Rapid Virus Passage. High viral titers in cell culture were needed in this study, therefore virus isolates were passed in cell cultures until titers exceeding eight logs

could be established. Depending on the isolate, either Ma-104 or ST cells were used to pass the different virus isolates. Confluent monolayers were rinsed three times with rotavirus maintenance medium (RMM) prior to inoculation. The medium, RMM, consisted of the cell growth medium described earlier with exception of the calf serum which was deleted. This medium was further supplemented with 1 M hepes buffer (Research Organics) and 10 mcg/ml trypsin. The solution was pH adjusted with a 10 M NaOH solution to 7.4. Leighton tubes with confluent coverslips were used for rapid passage work. One hundred-fold dilutions of virus-cell suspensions were made in RMM. The third rinse was removed from the tubes and they were incubated with 1.5 ml of virus fluids/tube. The tubes were incubated at 37°C for 24-48 hours which was dependent upon the observation of cytopathic effect (CPE). Once CPE was detected, the infected cells were frozen and thawed three times and then stored at -70°C. Each pass was assayed for virus titer when a titer greater than eight logs was reached, the rapid passage was discontinued.

Viral Titration. Titration of viral samples, to determine the number of infectious units, was done on monolayers of Ma-104 cells grown to confluency in 96-well microtiter plates. The plates were rinsed three times with RMM prior to inoculation. Ten-fold dilutions of the sample to be titrated were made in RMM. The rinse medium was removed

from the plates and five wells per dilution were inoculated with 0.1 ml volumes per well. Eight ten-fold dilutions were assayed per sample with the center two rows of the plate serving as uninoculated controls. Once inoculated, the plates were sealed and incubated at 37°C for 48 hours. The medium was removed and the plates were fixed in 80% cold acetone for 15 minutes at room temperature. Each titration assay was read by indirect immunofluorescent staining of the monolayers. Earlier studies indicated that these assays were reproducible if read between two to seven days post-inoculation. After fixation, the acetone was removed from the plates and they were stored at -20°C until stained.

Indirect Immunofluorescent Staining of Inoculated Cell Cultures. The fixed plates were rinsed with phosphate buffered saline (PBS) for 10 minutes prior to staining. The PBS was discarded and all the wells were covered with 0.05 ml of porcine rotavirus antiserum diluted 1/80 in PBS. This working dilution had previously been determined in positive fixed slides. The antiserum was prepared in gnotobiotic pigs and was monospecific for rotavirus. The plate was incubated in a humidified chamber at 37°C for 30 minutes. The rotavirus antiserum was removed and the plate was rinsed three times with PBS. The final rinse was removed and the plate was inoculated with 0.05 ml of rabbit antiporcine IgG FITC (Miles labs) diluted 1/80 in PBS. The plate was held in a humidified chamber for

30 minutes at 37°C. The conjugate was removed and the plate was rinsed three times with PBS. The plates were then inverted and the monolayers examined with a fluorescent microscope (American Optical model 2071). Wells were recorded as positive or negative for the presence of fluorescing cells. The tissue culture infectious dose 50% ($TCID_{50}$) was calculated for each sample using the Spearman-Karber method.

History of Rotavirus Isolates. All isolates with the exception of the Simian and vaccine strain had to be adapted to tissue culture. The viral pools reflected below all had titers exceeding eight logs.

(1) 056; This herd was located in Winfield, Iowa and rotavirus was isolated from a seven day old baby pig small intestinal extract (SIE). The isolate was passed four times in Ma-104 cells.

(2) Narigon; This herd was located in Nodaway, Iowa and rotavirus was isolated from a fourteen day old baby pig SIE. The isolate was passed four times in Ma-104 cells.

(3) 047; This herd was located in Fairmont, Minnesota and rotavirus was isolated from a three week old pig SIE. The isolate was passed four times in ST cells.

(4) Standard Challenge; A virulent OSU strain of porcine rotavirus was obtained from Dr. Bohl at Ohio Agricultural Research and Development Center. When received, the isolate had been passed several times in

gnotobiotic pigs. A new challenge was prepared by passage in gnotobiotic pigs and by making a 10% SIE from the infected pigs. Only one pass in pigs was needed as they developed clinical signs of a virulent rotavirus infection within 24 hours. This material was passed eight times in ST cells.

(5) TC-63; A commercial vaccine strain of porcine rotavirus was obtained from Ambico, Inc. This strain was originally obtained from Dr. Bohl and is also designated as OSU strain. The vaccine strain has been attenuated and has been found to be safe and efficacious in gnotobiotic pigs. This strain is easily grown in ST cells and only one passage was needed.

(6) Simian; A rhesus monkey strain, S:USA 79-2 was obtained from Dr. Woode at the College of Veterinary Medicine at Ames, Iowa. This strain served as a non-porcine rotavirus control. The isolate was highly cytopathic for Ma-104 cells and only had to be passed two times.

All isolates described above grew to titers exceeding eight logs in Ma-104 cells but only the 047, Std. Chall. and TC-63 isolates would grow in ST cells.

Preparation of Rotavirus Antigens for Inoculation of Guinea Pigs. Bulk viral pools of 400 ml were prepared for each virus isolate in confluent roller bottles. Either ST or Ma-104 cells were used depending on the isolates previous passage history. Three to five day old confluent roller

bottles were rinsed three times in RMM. The third rinse was removed and each of two roller bottles per isolate were inoculated with a 1/100 dilution of virus. The bottles were then incubated at 37°C on a roller rack (Belco) at 1.0 rpm for 24 hours. At the end of the incubation period all roller bottles demonstrated extensive cytopathic effect. The virus bulks were then pooled for each isolate and were frozen and thawed three times. The bulks were clarified of cellular debris by centrifugation at 5600 x g for one hour at 4°C (Sorval RC, 2-B). The supernatant fluids were then collected and stored at -20°C. The bulks were then centrifuged at 100,000 x g for 90 minutes at 4°C in a fixed angle rotor of a Beckman L5-65 ultracentrifuge. The supernatant fluids were discarded and the pellet was resuspended in 1.0 ml of PBS. This concentrated virus was layered onto 50% sucrose cushions and was recentrifuged as before, except a swinging bucket head was used for the ultracentrifugation. If at the end of the run tubes were cloudy, they were re-centrifuged. The supernatant fluids were discarded and all pellets resuspended in 1 ml volumes of PBS. All concentrated and purified viral bulks were sampled and examined by electron microscopy at Iowa State University Diagnostic Laboratory. All bulks contained numerous rotavirus particles with both complete and single-shelled particles being observed. To further identify the bulks as rotavirus they were assayed by the indirect fluorescent antibody staining procedure described earlier.

All bulks demonstrated positive cytoplasmic fluorescence in inoculated cell cultures.

Hyperimmunization of Guinea Pigs. Fourteen 300-500 gram guinea pigs (Sasco) were divided into seven groups: six groups for each of the six virus agents and one un-inoculated control group. All guinea pigs were bled by cardiac puncture prior to the first inoculation. The blood was allowed to clot and the serum portion was collected and heat inactivated at 56°C for 30 minutes. One ml of virus was added to an equal volume of Freund's incomplete adjuvant and was emulsified by passage through a 25 gauge needle until no separation was observed for 10 minutes. Simultaneous intramuscular and subcutaneous injections were given over four sites. The hyperimmunization schedule lasted for eight weeks consisting of five sets of injections. Starting with a 10^{-3} dilution of virus, the virus dilution decreased ten-fold until the final two sets of injections which were given undiluted. No adjuvant was used for the fifth injection and the volume was injected only intramuscularly. All guinea pigs were bled out one week after the fifth injection and serum portions were collected and processed as before. During the hyperimmunization schedule all groups were housed separately to prevent any cross-contamination due to oozing at the injection sites. Since there were two guinea pigs inoculated for each different virus isolate, serum samples

were pooled for each group to facilitate testing.

Neutralizing Antibody Assay. Each serum pool was tested for neutralizing antibody against all six virus isolates by a varying serum-constant virus assay. Eight two-fold dilutions were made in RMM for each serum sample to be tested. An equal volume of diluted virus (100-1000 $TCID_{50}$/tube) was added to each dilution. The virus-serum mixture was incubated at 37°C for 2 hours. Ma-104 cells grown to confluency in 96-well microtiter plates were used to assay the non-neutralized virus in the virus-serum mixtures. The monolayers were rinsed three times with RMM prior to inoculation. Five wells per dilution were inoculated with 0.1 ml volumes of the virus-serum mixture. The center two rows of the plate served as uninoculated controls, and were inoculated with 0.1 ml of RMM. The plates were sealed and placed at 37°C for two days at which time they were fixed, stained and read by indirect immunofluorescent staining procedure. The antiserum endpoints were calculated using the Spearman-Karber method. Serum titers were determined for each serum pool versus each different virus isolate.

Measurement of Cross-Neutralizing Antibody. Each hyperimmune antiserum was tested for its capability to neutralize all six different antigens. This was accomplished by using a varying virus-constant serum assay. The virus strain to be assayed was diluted in ten-fold fashion in

RMM. Eight dilutions were made per antigen sample. Each ten-fold dilution was split into seven, 0.8 ml aliquots. All seven antiserum pools were prediluted 1/100 in RMM. Each prediluted antiserum pool was added to equal volumes to one of the seven aliquoted virus dilutions. The serum-virus mixtures were incubated at 37°C for two hours. Ma-104 cells grown to confluency in 96 well microtiter plates were used to assay the non-neutralized virus. Five wells were inoculated per dilution with 0.1 ml volumes. The center two rows served as uninoculated controls and were inoculated with 0.1 ml of RMM. The plates were sealed and incubated at 37°C for 2 days at which time they were fixed, stained and read by the indirect immunofluorescent staining procedure. Wells were determined as positive if cells demonstrated cytoplasmic fluorescence. Virus titers were calculated for each serum-virus mixture by the Spearman-Karber method.

Plaque Inhibition Assay. Plaque inhibitions were performed using a varying virus-constant serum assay. The assay was similar in principle to the microtiter plate assay described above with two exceptions: the cell vessel inoculated was a 25 cm^2 flask and the determination of virus titer was calculated as Log plaque forming units per ml (Log_{10}PFU/ml). Only two virus isolates were used in this assay, the TC-63 (OSU strain) and the 056 (Iowa strain) isolates. These two isolates were chosen for their

reproducible plaquing characteristics. Three types of predilutions were used to assay the hyperimmune antiserums; 1/100, 1/50, and 1/20. Ten-fold virus dilutions were made in RMM and each dilution was distributed into six, 0.4 ml aliquotes. Each prediluted antiserum pool was added in equal volumes to one of the six aliquoted virus dilutions. The serum-virus mixtures were incubated for two hours at 37°C. Ma-104 cells were used to assay the Iowa (056) isolate and ST cells were used for the OSU (TC-63) isolate. Confluent cell cultures were rinsed three times with RMM prior to inoculation. The final rinse was poured off and the remaining volume removed with a pasteur pipette. Two flasks were inoculated per dilution with 0.2 ml/flask. The flasks were incubated at 37°C for 1 hour, with the serum-virus inoculums being rotated evenly over the cell sheet every 15 minutes. At the end of the incubation period, the inoculum was rinsed off three times with RMM. An 8 ml agar overlay was added to each flask. The overlay consisted of 3 parts RMM to 1 part 4% Noble agar (DIFCO). The solution was further supplemented with DEAE-Dextran (Sigma) to a 0.1% level. The agar was allowed to harden at room temperature and the flasks were inverted and incubated in the dark at 37°C for 4-7 days. At the end of the incubation period plaques averaging 3 mm in diameter were observed. All flasks were fixed with a 10% formalin-PBS solution for 15 minutes at room temperature. The agar was removed and the

cell sheets were stained with a 1% aqueous crystal violet solution for 10 minutes. The stain was removed and the flasks were rinsed with running tap water. The plaques appeared as clear holes in a darkly stained monolayer. The plaques were counted and Log_{10}PFU titers/ml calculated for each serum-virus mixture being assayed.

Calculation of the Neutralizing Index. A neutralizing index (N_i) was calculated for the neutralizing capacity of each hyperimmune antiserum. This was done by comparing the titer of the virus after treatment for 2 hours at 37°C with the antiserum (N_i) with the titer of the virus after a similar treatment with the control negative serum (N_o). The neutralizing index is as follows:

$$N = \frac{N_o}{N_i} \text{ ; thus } \log_{10}N = \log_{10}N_o - \log_{10}N_i$$

DATA AND DISCUSSION

All porcine rotavirus isolates were grown in tissue culture and purified. Each was identified as rotavirus by indirect immunofluorescent staining which identifies the group antigen. Both complete and single-shelled virus particles were detected in the purified virus preparations by electron microscopy. The presence of complete particles was significant because the outer capsid is the site of species-specific antigens (Bridger 1978). Hyperimmunization

with single-shelled particles will produce antiserum to group antigen only, whereas immunization with outer capsid proteins will produce species-specific antiserum as determined by serum neutralization studies (Estes et al. 1979; Matsuno and Inouye 1983).

Each of the six guinea pig hyperimmune sera prepared against each of the six rotavirus isolates (TC-63, Std. Chall., 047, Nar., 056, and the simian) were assayed in vitro for neutralizing antibody versus each of the rotavirus isolates using the constant virus-varying serum procedure. The results are given in Table 1. It is clear that the hyperimmunization was successful in producing high specific homologous titers. The generation of high titer hyperimmune antiserum is essential when comparing different rotavirus strains. In work done with human rotavirus, hyperimmune sera prepared against the three serotypes were essential to distinguish between them. It was found that post-infection sera had low titers and were more cross-reactive than hyperimmune sera (Wyatt et al. 1982).

On the basis of the results in Table 1, the six rotavirus isolates were divided into three groups:

(1) TC-63, Std. Chall., and 047 are similar and are assumed to belong to the OSU porcine strain of rotavirus, since both TC-63 and Std. Chall. were derived from OSU strain isolates provided by Dr. Bohl at OARDC.

Table 1. Hyperimmune Serum Titers: Porcine Rotavirus Neutralizing Antibody Titers versus Homologous and Heterologous Rotavirus Isolates

Rotavirus Antigens (Strain)/Code	Pre-Vac Titers	Anti-OSU Strain Porcine Rotavirus			Anti-Iowa Strain Porcine Rotavirus		Anti-Simian Rotavirus
		Anti-TC-63	Anti-Std. Chall.	Anti-047	Anti-Nar.	Anti-056	
Porcine (OSU)/TC-63	49	11,900	1,300	13,700	197	227	393
(OSU)/SC	45	5,970	1,710	27,400	227	171	137
(OSU)/047	57	4,520	1,750	11,900	227	227	686
Porcine (Iowa)/Nar.	80	299	227	299	18,100	18,100	299
(Iowa)/056	86	149	149	130	7,870	9,040	343
Simian/Sim	100	113	113	113	452	227	31,400

(2) Nar. and 056 are similar, but are shown to be distinctly different from the OSU porcine rotavirus. Therefore, they were designated the Iowa strain of porcine rotavirus.

(3) The simian rotavirus is distinct and different from either the OSU or the Iowa strains of porcine rotavirus, confirming the species-specificity of the reactions observed.

To confirm these results, each of the six guinea pig hyperimmune sera were tested in vitro for their ability to neutralize each of the six rotavirus isolates. The constant serum-varying virus procedure was utilized and neutralizing indices calculated for each combination of rotavirus isolate and hyperimmune serum. The results are presented in Table 2a which represents the titers with and without treatment with each hyperimmune serum. Table 2b is a summary of the neutralizing indices as calculated from the results in Table 2a. Three groups of rotavirus are evident confirming the results in Table 1:

(1) TC-63, Std. Chall. and 047 are shown to be similar to the OSU strain of porcine rotavirus.

(2) Nar. and 056 are shown to be similar and distinctly different from the OSU strain, therefore, this strain was designated the Iowa strain. The Iowa strain is designated as a porcine rotavirus based upon its isolation from two separate herds of swine in Iowa. The relationship of this

Table 2a. Rotavirus Titers With and Without Treatment with Hyperimmune Sera, Using Fluorescent Antibody Virus Assay System: $Log_{10}TCID_{50}/ml$

Rotavirus Antigens (Strain)/Code	Anti-OSU/Porcine Rotavirus						Anti-Iowa/Porcine Rotavirus					
	Anti-TC-63		Anti-SC		Anti-047		Anti-Nar		Anti-056		Anti-Simian	
	(-) N_o	(+) N_i	(-) N_o	(+) N_i	(-) N_o	(+) N_i	(-) N_o	(+) N_i	(-) N_o	(+) N_i	(-) N_o	(+) N_i
Porcine (OSU)/TC-63	6.53	$\leq$1.20	6.53	$\leq$1.20	6.53	$\leq$1.20	6.87	6.33	6.53	6.33	6.87	6.53
(OSU)/SC	6.20	$\leq$1.20	5.53	$\leq$1.20	6.33	$\leq$1.20	6.20	5.20	6.33	5.33	6.20	5.87
(OSU)/047	6.20	$\leq$1.20	6.53	$\leq$1.20	6.20	$\leq$1.20	6.33	5.87	6.33	5.20	6.53	6.08
Porcine (Iowa)/Nar	5.87	5.08	6.08	5.87	6.53	6.08	6.08	$\leq$1.20	5.53	$\leq$1.20	5.87	5.53
(Iowa)/056	5.08	4.87	5.08	4.87	5.53	5.08	5.33	$\leq$1.20	5.33	$\leq$1.20	5.33	4.53
Simian/Sim	5.97	5.08	6.08	5.20	5.87	4.87	6.08	5.20	5.87	5.87	5.53	$\leq$1.20

N_o = Log_{10} $TCID_{50}/ml$ Fluorescent Antibody-Virus Titer Following Treatment with Pre-Vac, Negative-Control serum (1/100 dilution).

N_i = Log_{10} $TCID_{50}/ml$ Fluorescent Antibody-Virus Titer Following Treatment with Hyperimmune Serum Prepared against Designated Rotavirus Isolate (1/100 dilution).

Table 2b. Neutralization Indices Determined for each Combination of Homologous and Heterologous Antigens and Antisera, from Data in Table 2a. Log_{10} Neutralization Indices (N)

Rotavirus Antigens (Strain)/Code	Anti-OSU/Porcine Rotavirus			Anti-Iowa/Porcine Rotavirus		Anti-Simian
	Anti-TC-63	Anti-SC	Anti-047	Anti-Nar	Anti-056	
Porcine (OSU)/TC-63	≥5.33	≥5.33	≥5.33	0.54	0.20	0.34
(OSU)/SC	≥5.00	≥4.33	≥5.33	1.00	1.00	0.33
(OSU)/047	≥5.00	≥5.33	≥5.00	0.46	1.13	0.45
Porcine (Iowa)/Nar	0.79	0.21	0.45	≥4.88	≥4.33	0.34
(Iowa)/056	0.21	0.21	0.45	≥4.13	≥4.13	0.80
Simian/Sim	0.79	0.88	1.00	0.88	0.00	≥4.33

strain with other nonsimian species of rotavirus has not been studied.

(3) The third group of rotaviruses evident in Tables 2a and 2b is the simian group, which has its own unique neutralizing index, differing from both the OSU and Iowa strains of porcine rotavirus.

All three groups described above were compared using the students t-test to determine statistical difference between two independent means. All three groups were found to be significantly different from each other with t-values greater than those calculated at the 0.001 alpha level of significance.

Plaque inhibitions were performed in order to further compare the OSU strain with the Iowa strain for minor antigenic similarities. Each of the five guinea pig hyperimmune sera prepared against the five porcine rotavirus isolates, were tested for their ability to neutralize an OSU isolate (TC-63) or an Iowa isolate (056). The OSU strain was plaqued in ST cells, while the Iowa strain was plaqued in Ma-104 cells. The titer of each virus strain was determined after treatments with pre- and post-hyperimmune sera. The varying virus-constant serum assay was used at three predetermined dilutions of serum (1/20, 1/50 and 1/100). This was done in order to determine whether there were antibodies to a secondary antigen present at low levels in the serum samples. The problem

associated with non-specific neutralization of the virus when added to these low dilutions of sera was minimized by rinsing off the virus-serum inoculum with RMM following adsorption of the virus. This effectively eliminated non-specific inhibitors of rotavirus in the serum. The results are given in Table 3a (for the OSU strain) and in Table 4a (for the Iowa strain). The neutralization indices were calculated for each set of results and are presented in Tables 3b and 4b, respectively. The results indicated a low level of neutralizing antibody in the anti-Iowa hyper-immune sera versus the OSU strain of rotavirus at a low dilution (1/20, Table 3b). Low levels of neutralizing antibody were also found in the anti-OSU hyperimmune sera versus the Iowa strain of rotavirus (Table 4b). These results indicated a minor antigenic similarity between the two strains of porcine rotavirus while confirming the absence of a major antigenic similarity.

The compiled results indicate a low level of cross-neutralizing antibody in all three groups. The amount of cross-neutralizing antibody found in the simian sera to the OSU strain is similar to the results reported previously by others (Woode et al. 1982).

Other antigenically distinct porcine rotaviruses have been identified by electron microscopy. They have been designated as porcine rotavirus-like agents or as pararotavirus agents (Bohl et al. 1982; Bridger et al. 1982).

Table 3a. Rotavirus Titers of TC-63/OSU/Rotavirus with Pre-Vac, Homologous and Heterologous Hyperimmune Sera, Using Plaque Assay System: Effect of Dilution of Hyperimmune Sera. Log_{10} PFU/ml

	Anti-OSU/Porcine Rotavirus				Anti-Iowa/Porcine Rotavirus	
	Anti-TC-63		Anti-SC	Anti-047	Anti-Nar	Anti-056
	(-)	(+)	(+)	(+)	(+)	(+)
Dilution of Serum	N_o	N_i	N_i	N_i	N_i	N_i
1/100	7.57	3.74	ND[1]	ND	7.10	7.35
1/100	7.05	3.88	4.97	ND	6.82	6.73
1/50	7.69	4.03	5.38	3.69	6.70	6.40
1/50	7.51	2.70	4.48	2.00	5.58	5.90
1/20	7.87	1.00	3.15	1.00	4.00	5.40
1/20	7.74	1.00	ND	ND	5.16	5.39

N_o = Log_{10} PFU/ml following treatment with Pre-Vac negative control serum.

N_i = Log_{10} PFU/ml following treatment with Hyperimmune serum prepared against designated rotavirus isolate.

ND[1] = Not Done

Table 3b. Neutralization Indices of TC-63/OSU/Rotavirus Following Treatment with Homologous and Heterologous Hyperimmune Sera, Determined from Data in Table 3a: The Effect of Dilution of the Hyperimmune Serum on Virus Neutralization. Log_{10} Neutralization Indices (N)

Dilution of Serum	Anti-OSU/Porcine Rotavirus			Anti-Iowa/Porcine Rotavirus	
	Anti-TC-63	Anti-SC	Anti-047	Anti-Nar	Anti-056
1/100	3.83	ND[1]	ND	0.47	0.22
1/100	3.17	2.08	ND	0.23	0.32
1/50	3.66	2.31	4.00	0.99	1.29
1/50	4.81	3.03	5.51	1.93	1.61
1/20	6.87	4.72	6.87	3.87	2.47
1/20	6.47	ND	ND	2.31	2.08

ND[1] = Not Done

Table 4a. Rotavirus Titers of 056/Iowa/Rotavirus with Pre-Vac, Homologous and Heterlogous Hyperimmune Sera, Using Plaque Assay System: Effect of Dilution on Hyperimmune Sera. Log_{10} PFU/ml

	Anti-OSU/Porcine Rotavirus			Anti-Iowa/Porcine Rotavirus		
	Anti-TC-63	Anti-SC	Anti-047	Anti-Nar	Anti-056	
	(+)	(+)	(+)	(+)	(-)	(+)
Dilution of Serum	N_i	N_i	N_i	N_i	N_o	N_i
1/100	5.40	6.30	6.53	2.40	7.29	2.40
1/100	4.70	6.38	5.81	2.00	6.63	2.00
1/50	5.08	ND[1]	ND	2.24	7.23	2.48
1/50	4.94	5.94	5.81	1.88	7.44	2.35
1/20	3.87	4.78	4.50	1.00	6.35	1.40

N_o = Log_{10} PFU/ml following treatment with Pre-Vac, negative control serum.

N_i = Log_{10} PFU/ml following treatment with hyperimmune Serum Prepared against designated rotavirus isolate.

ND[1] = Not Done.

Table 4b. Neutralization Indices of 056/Iowa/Rotavirus Following Treatment with Homologous and Heterologous Hyperimmune Sera, Determined from Data in Table 4a: The Effect of Dilution of the Hyperimmune Serum on Virus Neutralization. Log_{10} Neutralization Indices (N)

Dilution of Serum	Anti-OSU/Porcine Rotavirus			Anti-Iowa/Porcine Rotavirus	
	Anti-TC-63	Anti-SC	Anti-047	Anti-Nar	Anti-056
1/100	1.89	0.99	0.76	4.89	4.89
1/100	1.93	0.25	0.82	4.63	4.63
1/50	2.15	ND[1]	ND	4.99	4.75
1/50	2.50	1.50	1.63	5.56	5.09
1/20	2.48	1.57	1.85	5.35	4.95

ND[1] = Not Done

In both cases the virus isolates did not cross-react with rotavirus group antigen as determined by immunofluorescence. Thus both isolates studied are not truly rotavirus isolates, because it has been demonstrated that all rotavirus isolates will contain a common group antigen. Therefore the significance of this study is based upon the identification of a new and unique strain of porcine rotavirus, one that has a similar group antigen yet possessing distinct major neutralizing antigens. Furthermore, this study opens the possibility of the existence of other serologically distinct porcine rotaviruses. The demonstration of these serologically distinct porcine rotaviruses are important in the development of effective vaccines for the control of this ubiquitous porcine pathogen.

CONCLUSIONS

Hyperimmune guinea pig sera with relatively high antibody titer was essential, in order to distinguish between rotavirus isolates. Two porcine strains and one simian strain of rotavirus were identified. The simian strain lacked any major serotypic relationship to the two porcine strains of rotavirus. The porcine rotavirus isolates: TC-63, Std. Chall. and 047 have been shown to be similar and are assumed to be the OSU strain of porcine rotavirus. Two of these, TC-63 and the Std. Chall., were derived from the OSU strain. The Narigon and 056 porcine rotavirus

isolates are designated the Iowa strain and are different from the OSU strain on the basis of major antigenic differences. Plaque inhibition studies using varying dilutions of hyperimmune sera, suggest low titer minor antigenic similarities between the OSU and Iowa strains, while confirming the absence of any major serotypic correlation.

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